{Exhibit 40}

Schott, H., et al., "A Dihydroxyboryl-Substituted
Methacrylic Polymer for the Column
Chromatographic Separation of Mononucleotides,
Oligonucleotides, and Transfer Ribonucleic Acid,"
Biochemistry 12: 932-937 (1973)

A Dihydroxyboryl-Substituted Methacrylic Polymer for the Column Chromatographic Separation of Mononucleotides, Oligonucleotides, and Transfer Ribonucleic Acid[†]

Herbert Schott, Eberhard Rudloff, Peter Schmidt,
Ranajit Roychoudhury, and Hans Kössel*

ABSTRACT: The properties of a new dihydroxyboryl-substituted methacrylic acid polymer ("borate gel") and its application to the column chromatographic separation of nucleic acids components are described. Due to complex formation between the borate groups and the cis diol groups, ribonucleoside 5'-phosphates, 3'-ribonucleoside-terminated oligodeoxynucleotides, and free tRNAs are absorbed at high salt concentrations at pH 8.5, whereas deoxynucleoside phosphates, ribonucleoside 2'- or 3'-phosphates, oligodeoxynucleotides, and aminoacyl-tRNAs are eluted within the void volumes of the borate gel columns. The diol–borate complexes can be dissociated by lowering the pH and the salt concentration of the eluent, releasing the absorbed compounds from the column. Crude tRNA isolated by standard procedures can be further purified by removal of unchargeable polynucleotide. The separation of free tRNA from aminoacyl-tRNA opens a new approach for the isolation of any set of isoaccepting tRNA species from bulk tRNA.

The complex formation of dihydroxyboryl groups with cis diol groups of ribonucleosides and ribonucleoside 5'-phosphates has been used as a basis for the separation of these compounds in paper chromatographic and paper electrophoretic systems on an analytical scale (for a review, see Khym, 1967). Subsequently, this principle was applied to preparative column chromatographic procedures by using as matrices cellulose (Weith *et al.*, 1970) or poly(methacrylic acid) gels (Schott, 1972a,b), to which dihydroxyboryl groups had been attached covalently. The chemical constitution of the latter is represented as:

$$\left[\begin{array}{c} H \quad CH_3 \\ | \quad\quad | \\ -C-C- \\ | \quad\quad | \\ H \quad\quad C \\ \quad\quad O \end{array} \right]_n$$
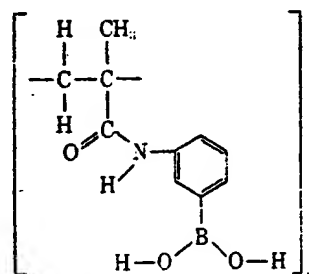
It has been demonstrated that on columns of both such types, ribonucleosides but not deoxyribonucleosides are retained at slightly alkaline pH; on the borate-containing cellulose, oligonucleotides derived from the 3' terminus of phage RNA bearing 3'-terminal cis diol groups can be separated from the corresponding internal RNA fragments terminating in 3'-phosphomonoester groups (Rosenberg and Gilham, 1971). However, no such information has been reported for the dihydroxyboryl-substituted methacrylic acid polymer.

In order to allow a comparison of the two borate-containing matrices with respect to resolution power, capacity, and recovery of the separated compounds, a more detailed study of the basic absorption properties of the new type of poly(methacrylic acid) gel seemed desirable. Secondly, the question arose as to whether the list of separable compounds could be extended from nucleosides to mono- and oligonucleotides and to polynucleotides of higher chain length, such as tRNA and DNA. A third principal aim of the present study was to investigate the applicability of the borate gel for the separation of aminoacylated tRNA from free tRNA. The former lacks 3'-terminal cis diol group and should therefore appear within the void volume of the gel, whereas the nonaminoacylated tRNA should be retained or eluted only after lowering the pH and/or the salt concentration of the eluent.

In this article evidence is presented that separation of ribonucleoside 5'-phosphates from 2'- or 3'-phosphates and from deoxymononucleotides is readily achieved on a larger scale by column chromatography on the borate gel.[1] In addition, synthetic ribonucleoside-terminated oligodeoxynucleotides obtained by chemical synthesis or resulting as final products from the 3'-terminal labeling of oligodeoxynucleotides (Kössel and Roychoudhury, 1971) can be separated satisfactorily from deoxynucleoside 5'-monophosphates, oligodeoxynucleotides, or polydeoxynucleotides on both preparative and analytical scales. Furthermore, conditions have been found under which complex formation effectively separates uncharged tRNA from aminoacylated tRNA, though the alkali lability of the aminoacyl residues on tRNA was envisaged as a possible major problem of this new technique.

Materials and Methods

Nucleotides. Nonradioactive mononucleotides were purased from Papierwerke Waldhof-Aschaffenburg (Mannim, Germany).

Oligonucleotides. A mixture of oligothymidylic acids. p-T)$_n$, and of oligothymidylic acids terminated with ribourine residues at the 3' ends, p(dT)$_n$U, was prepared by mixed ilycondensation of 5'-thymidylic acid and (2',3')-O-dietyluridine 5'-phosphate in the presence of mesitylene-lfonyl chloride according to the following procedures.

Pyridinium pdT (805 mg; 2.1 mmol) and 447 mg of pyridinm prU(OAc)$_2$ (0.8 mmol) in 5 ml of dry pyridine were alwed to react with 1 g (4.5 mmol) of mesitylenesulfonyl chioie for 4 hr at room temperature. Then 7.5 ml of water was ided in the cold. The mixture was kept at room temperature r 2 hr and then treated with 25 ml of concentrated ammonia ernight in order to hydrolyze the O-acetyl groups. After reoval of ammonia by evaporation, the total solution was adsted to 5 mM Et$_3$NH$_2$CO$_3$ and 20% methanol in a final volne of 250 ml and subjected to column chromatography on EAE-cellulose as shown in Figure 1. Characterization of the icleotide material from the peaks of Figure 1 was carried it by paper chromatography and by determination of the icleoside/nucleotide ratios of the purified compounds by gradation with snake venom phosphodiesterase after reoval of the 5'-terminal phosphomonoester group with alkaie phosphatase. Thus peaks C and D were identified as unacted pdT and prU, respectively, while pdT-dT and pdT-J appeared in peaks G and H. The double peak N–O conining the pentanucleotides shows only a partial resolution ? p(dT)$_5$ from p(dT)$_4$-rU; paper chromatographic analysis dicated that the two compounds were present in roughly jual amounts. p(dT)$_4$-rU after paper chromatographic paration followed by treatment with alkaline phosphatase lowed the same R_F values as (dT)$_4$-rU prepared on a differit synthetic route by block condensation of (MeOTr)dT-dT-T and pdT-rU(OAc)$_2$ (Schmidt and Kössel[2]); degradation of IT)$_4$-rU with snake venom led to the ratio of dT:pdT:prU : 1.04:3.08:1.00 (calcd, 1:3:1).

d(A-C-C-A-T-T-C-A)[^{32}P]prU was prepared by the terinal addition of a [^{32}P]prU residue to the synthetic octaucleotide d(A-C-C-A-T-T-C-A) (Schott and Kössel[3]) in the resence of terminal deoxynucleotidyl transferase and [α-^{32}P]TP (specific activity 1–3 Ci/mmol) essentially as described or the 3'-terminal end-group labeling technique for oligoeoxynucleotides (Kössel and Roychoudhury, 1971; Roynoudhury, 1972).

Low molecular weight DNA was obtained by limited digeson of calf thymus DNA with micrococcal nuclease (Hurwitz nd Novogrodsky, 1968).

tRNA. Stripped *Escherichia coli* tRNA was prepared acording to Zubay (1962). Preparation of *E. coli* aminoacyl-RNA synthetases and of radioactive aminoacyl-tRNA from . coli was carried out according to Muench and Berg (1966), xcept that the hydroxylapatite chromatography was omitted or the enzyme preparation and the 0.28 M KCl fractions from)EAE-cellulose. after concentration by dialysis. were used as nuxtures containing all the aminoacyl-tRNA synthetases. tadioactive amino acids with the specific activities of 4760 :i mol ([^{3}H]lysine). 310 Ci mol ([^{14}C]lysine), 4800 Ci mol ^{3}H]glutamic acid). and 260 Ci mol ([^{14}C]glutamic acid) were

[2] Schmidt, P., and Kössel, H., unpublished data.
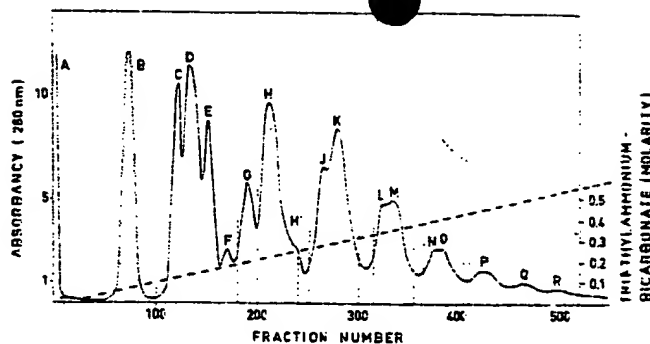[3] Schott. H., and Kössel, H., manuscript in preparation.

FIGURE 1: Separation of an oligonucleotide mixture resulting from mixed polycondensation of 5'-thymidylic acid and 2',3'-O-diacetyl-5'-uridylic acid by chromatography on a DEAE-cellulose column (42 cm × 2.5 cm bicarbonate form). Elution was carried out in the cold with a linear gradient using 5 l. of 0.02 M Et$_3$NH$_2$CO$_3$–20% methanol in the mixing vessel and 5 l. of 0.6 M Et$_3$NH$_2$CO$_3$–20% methanol in the reservoir. The material from the various peaks was characterized by the 280/260 and 250/260 ratios and by paper chromatography (see Materials and Methods).

bought from Schwarz Biochemical Corporation (Orangeburg, N. Y.). For large scale preparations, tRNA after aminoacylation was purified and concentrated by immediate absorption on 0.5-ml DEAE-cellulose columns; after washing with 10 ml of buffer A,[1] the mixtures of aminoacylated and free tRNAs were eluted with 2 ml of buffer B.[1]

Buffers and Eluents. Triethylammonium bicarbonate (1 M) of pH 8.5, prepared according to Ralph and Khorana (1961), was used as solvent for the absorption of cis diol-containing mono- and oligonucleotides. Desorption of the latter compounds was achieved by elution with double-distilled water (pH 5–6). Buffers A[1] and B[1] were used for purification and concentration of tRNA on DEAE-cellulose columns. Loading of the gels with mixtures of charged and uncharged tRNA and elution of aminoacyl-tRNA was carried out in the presence of buffer C.[1] Elution of uncharged tRNA was achieved with buffer D.[1] High magnesium chloride concentration seems essential at least in buffer C, as in test runs according to Figure 5, but with 0.01 M MgCl$_2$ only a trace amount of tRNA was absorbed to the column. Buffers C and D show absorptions at 260 nm in the range of 0.08–0.16 (buffer C) and 0–0.05 (buffer D), which are not subtracted in Figures 5 and 6.

Preparation and Capacity of the Borate Gels. The gels were prepared essentially as described by Schott (1972a,b). Treatment of the polymer with acetone is essential in order to optimize the gel with respect to the flow rate. From 1 g of dry polymeric powder, 6 ml of wet gel was obtained. Elementary analysis performed by A. Bernhardt (Elbach, Germany) showed: C. 61.60%; H, 7.87%; N, 1.13%; and variations of the boron content from 0.11 to 0.58%. Low boron content (0.11%) of the gel was observed when the polymerization reaction was carried out with higher concentrations of cross-linking reagent for a prolonged time period (24 hr instead of 16 hr). The capacity of various gel preparations to retain uncharged tRNA at pH 8.5 was measured by passing an excess of tRNA dissolved in buffer C (e.g., 3 ml of a tRNA solution containing 90 A_{260} units/ml of tRNA for a 25-ml column) followed by elution with buffer C until the absorbancy at 260 nm decreased to a constant level lower than 0.2; the totally absorbed tRNA was then eluted with buffer D and estimated spectrophotometrically. The capacity in respect to mononucleotides was measured in the same way except that Et$_3$NH$_2$CO$_3$ and water were used instead of the buffers C and D. The capacity estimated by this
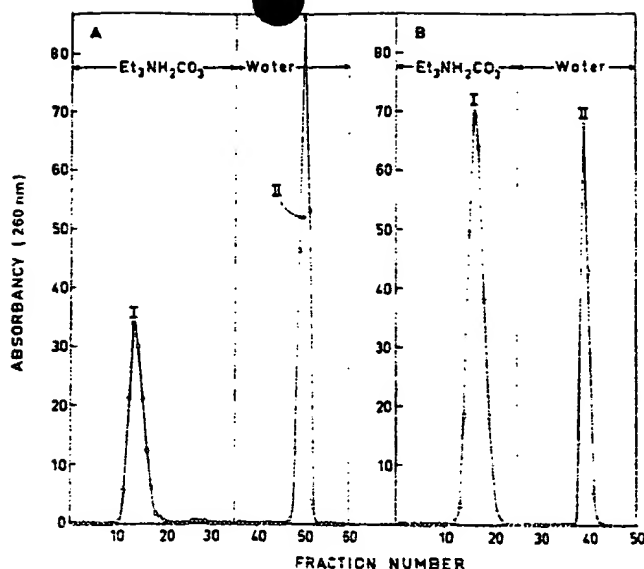
FIGURE 2: Column chromatographic separation of mononucleotides on borate gel. A mixture of the four standard ribonucleoside 5'-monophosphates (340 A_{260} units) and of the four deoxyribonucleoside 5'-monophosphates (190 A_{260} units) was applied in A. In B. a mixture of 16 mg of ribocytidine 5'-phosphate and 13 mg of riboadenosine 2'(3')-phosphate was chromatographed. Fractions of 1.3 ml were collected every 4 min from 25 cm $\times$ 1 cm columns. The elution buffers are indicated by the arrows. By paper chromatographic (A) or spectral (B) analysis, material from the peaks was identified as a mixture of the four deoxyribonucleoside 5'-monophosphates (AI), a mixture of the four ribonucleoside 5'-phosphates (AII), riboadenosine 2'(3')-phosphate (BI), and ribocytidine 5'-phosphate (BII).

procedure for tRNA showed almost linear correlation to the boron content; thus gels containing 0.58, 0.44, and 0.11% boron absorbed 80, 55, and 20 A_{260} units of tRNA per 25-ml bed volume, respectively. The capacity of a similar column in respect to 5'-riboadenylic acid was 680 A_{260} units. Flow rates in the range of 30–50 ml/hr were observed consistently when columns of 30-cm length and 1-cm width (25–30 ml) were used with a hydrostatic pressure corresponding to a difference of 1 m of water level. Peristaltic pumps are therefore not necessary. The flow rates and capacities did not change significantly when the same columns were used repeatedly without repacking.

Column Chromatographic Procedures. All column runs were performed at 4°. Mixtures of mono- and oligonucleotides dissolved in 1 M $Et_2NH_2CO_3$ were applied on top of the columns preequilibrated with the same solvent. Elution with 1 M $Et_2NH_2CO_3$ was then carried out until the first nucleotide peak had passed and the absorbancy recorded by an ultraviolet monitor had reached a constant low level of less than 0.1. Subsequently, the cis diol containing nucleotides of oligonucleotides were released from the gels by elution with distilled water. Recovery of the A_{260} units ranged between 90 and 100%. Unsatisfactory resolution was encountered consistently when the nucleotidic material was applied to the columns in unbuffered aqueous solution instead of $Et_2NH_2CO_3$.

For separation of aminoacylated tRNA, up to 30 A_{260} units of mixtures of aminoacylated and free tRNA's in 2 ml were first dialyzed against 0.01 M sodium acetate (pH 4.5). Then one-ninth of the volume of buffer containing 0.5 M morpholine·HCl (pH 8.5), 0.1 M $MgCl_2$, and 1 mM 2-mercaptoethanol was added in the cold and the resulting mixtures were instantly applied to the columns and eluted with buffer C.

Aminoacyl-tRNA usually appeared within 1–2 bed volumes In order to minimize hydrolysis of the aminoacyl residues the aminoacyl-tRNA-containing fractions were immediately brought to a neutral pH by the addition of 200 µl of 1 M sodium acetate (pH 4.5) per 5-ml fraction. Uncharged tRNA was then eluted with buffer D. Concentration of tRNA from the isolated peaks was carried out by absorption on small DEAE-cellulose columns (0.5-ml bed volumes) followed b elution with 2 ml of buffer B. Recovery of the A_{260} units fo tRNA varied in the range of 75–85%. Purification of bulk tRNA from unchargeable RNA was performed in the same way except that the dialysis step was omitted and the tRNA dissolved in buffer C was applied to the column directly; in thi case neutralization of the fractionated material with sodium acetate also was not necessary.

Paper Chromatography. For identification and in order to check cross-contamination of the cis diol-containing compounds with deoxynucleotides or ribonucleotides bearing 2'- or 3'-phosphomonoester groups, paper chromatography in the system isopropyl alcohol–concentrated ammonia–0.1 M boric acid (7:2:1; v/v/v) was used throughout with th descending technique on Schleicher-Schüll paper No. 2040b o 2043b.

Counting of Radioactivity. In order to determine the radio activity of mononucleotide- or oligonucleotide-containing fractions, aliquots of the various fractions were placed o filter paper disks, air-dried, and counted in a Beckmann liqui scintillation spectrometer. In the case of [14C]aminoacyl tRNA, acid-precipitable radioactivity was measured b washing the filter paper disks in cold 5% trichloroacetic aci as described by Mans and Novelli (1961). [3H]Aminoacyl tRNA was determined according to Waters and Novelli (1968).

Results and Discussion

Separation of Mononucleotides and Oligonucleotides. Figure 2 shows the separation of ribomononucleotides carrying 2 or 3' cis diol groups from the corresponding deoxynucleosid 5'-phosphates and ribonucleoside 2'- or 3'-monophosphate on a preparative scale. Material from the various peaks, whe tested by paper chromatography, in all cases showed negligibl if any cross-contamination from the neighboring peak: Recovery of the A_{260} units was 99 $\pm$ 5% (Figure 2A) an 100 $\pm$ 5% (Figure 2B), respectively. Quantitative separation c the two components applied in the case of Figure 2B was als evident from the observed spectral ratios of the two peak: thus peak I of Figure 2B showed an A_{250}/A_{260} ratio of 0.1 (calcd 0.15) and an A_{250}/A_{260} ratio of 0.79 (calcd 0.80), bot characteristic for the adenylate chromophore, whereas in pea II of Figure 2B, an A_{280}/A_{260} ratio of 0.99 (calcd 0.98) and a A_{260}/A_{260} ratio of 0.84 (calcd 0.84), both characteristic for th cytidylate chromophore, were observed throughout th fractions.

Figure 3 shows a separation of p(dT)$_5$ from p(dT)$_4$-rU on borate gel column, also on preparative scale; from the 200 A_{260} units applied to the column, 74 A_{260} units were found in peak [p(dT)$_5$] and 125 A_{260} units were isolated in peak II [p(dT) rU]. Cross-contamination of material from the two peaks wa exlcuded by paper chromatography. As both the olig nucleotides are of the same chain length and carry essentiall the same net charge at any pH, separation on preparative sca would not be possible by the usual chromatographic or ele trophoretic procedures, as is evident from the poor resolutio of the two compounds in the double peak N–O of Figure 1. O
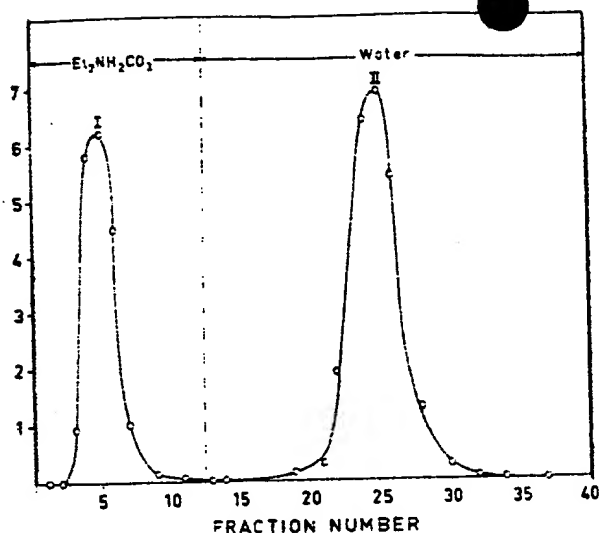
FIGURE 3: Column chromatographic separation of p(dT)₅ from p-dT)₄-rU on borate gel. 200 A_{260} units from peak N–O of Figure 1 after removal of the solvent were dissolved in 1.5 ml of 1 M Et₃NH₂CO₃ and applied to the borate gel column (15 cm × 1.5 cm). Elution was carried out with the buffers indicated by the arrows. Fractions of 4.5 ml were collected every 2–3 min.

he basis of the borate complex formation with the 3'-terminal ribouridine residue of p(dT)₄-rU, borate columns may be the only choice for the resolution of such compound mixtures on a large scale.

Figure 4 shows the analytical scale separation of an oligodeoxynucleotide with a 3'-terminal ribonucleotide residue from DNA on a borate gel column. Cross-contamination is excluded by the absence of ³²P radioactivity from peak I and by the absence of ultraviolet absorbing material from peak II. The recovery of material in terms of counts per minute was 86% and apparently all of the 2.7 A_{260} units of DNA applied could be regained in peak I. Analogous results were obtained, when the same ribonucleoside terminated oligodeoxynucleotide was chromatographed together with [¹⁴C]dATP (not shown). The addition of ribonucleotide residues to the 3' terminus of an oligodeoxynucleotide catalyzed by the enzyme terminal transferase was recently developed as a new end group labeling technique for oligodeoxynucleotides (Kössel and Roychoudhury, 1971; Kössel et al., 1973). Purification of the oligodeoxynucleotides carrying a ribonucleoside residue at the 3' terminus from side products (as, e.g., unreacted oligodeoxynucleotide and/or radioactive inorganic phosphate liberated from the ribonucleoside [³²P]-triphosphates used for the labeling procedure) should be facilitated by the new technique described here, especially when preparations of ribonucleoside-terminated oligodeoxynucleotides larger than can be purified by paper chromatography or by the two-dimensional fingerprint technique are required. In particular, for quantitative hybridization assays with radioactive ribonucleoside-terminated oligodeoxynucleotides (Fischer and Kössel[4]), the absence of unreacted oligodeoxynucleotide is essential.

In contrast to the nonvolatile buffers introduced for separation on borate cellulose by Rosenberg and Gilham (1971), which in this work also proved useful for tRNA separation (see below), the volatile Et₃NH₂CO₃ buffer and
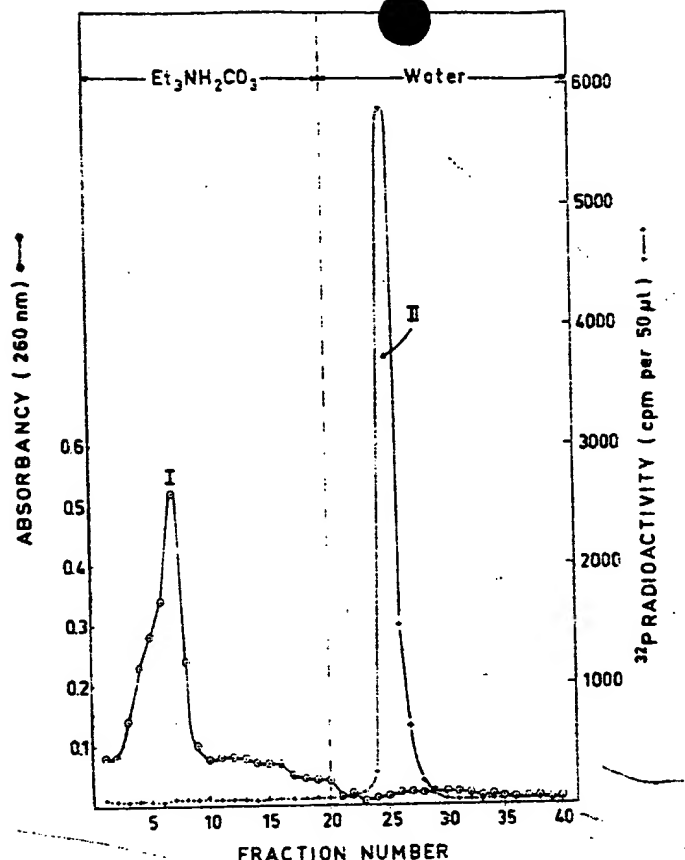

FIGURE 4: Column chromatographic separation of a radioactively labeled ribonucleotide-terminated oligodeoxynucleotide from DNA. The nucleotide mixture (2.7 A_{260} units of partially digested calf thymus DNA and 30,000 cpm of d(A-C-C-A-T-T-C-A)[³²P]prU) dissolved in 100 μl of 1 M Et₃NH₂CO₃, was, by means of a syringe, applied to the capillary column (6.5 cm long, 350 μl bed volume) and eluted as indicated by the arrows; three drop fractions were collected every 3–5 min; 50 μl of each fraction was counted on paper disks for radioactivity. The absorbancy was measured after diluting 100-μl aliquots of each fraction with water to 500 μl. Filling of the column with the gel was achieved by suction.

distilled water, used in this study for the separation of mono- and oligonucleotides, gave satisfactory results.

Purification of Transfer RNA. When crude tRNA from *E. coli* was chromatographed on a borate gel column (Figure 5), roughly one-half of the material was obsorbed at pH 8.5 and was eluted as a sharp peak (peak II) when the pH was lowered to 5.5, while the other half appeared within the void volume at pH 8.5 (peak I). Rechromatography of the isolated material from the peaks (not shown) showed complete reproducibility of the positions in the elution patterns, indicating that the appearance of peak I is not due to overloading of the column. When the isolated material, after concentration, was tested for aminoacylation, only material from peak II showed acceptor activity for lysine and glutamic acid, whereas material from peak I apparently consisted of contaminating polynucleotides different from tRNA (not shown). It seems unlikely that material from peak I represents aminoacylated tRNA or peptidyl-tRNA as the tRNA has been exposed to stripping conditions during the preparation. Thus the exact nature of this contaminant is not clear; no further effort was undertaken for its more thorough characterization. Similar contaminants in roughly the same quantity were, however, observed with different batches of *E. coli* tRNA and with tRNA from rabbit liver prepared according to Yang and

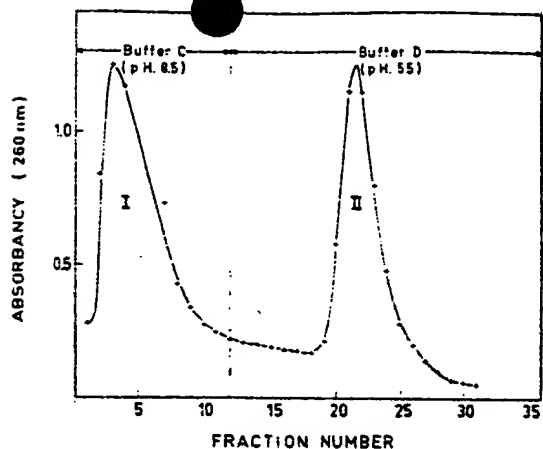4 Fischer, D., and Kössel, H., unpublished data.

FIGURE 5: Purification of crude *E. coli* tRNA from unchargeable polynucleotides by column chromatography on borate gel; 90 A_{260} units of tRNA dissolved in about 1 ml of buffer C was applied to the column (25 cm × 1 cm) and elution was carried out with buffers C and D as indicated by the arrows with a flow rate of 50–60 ml/hr; 4.6 ml was collected in each fraction.

Novelli (1968). For further purification, crude tRNA was therefore passed routinely through borate gel columns as in Figure 5 and only material corresponding to peak II was used for the method described subsequently.

Figure 6 shows a column chromatographic separation of aminoacylated tRNA from uncharged tRNA on borate gel. In both the cases tested, aminoacyl-tRNA lacking the terminal cis diol group appeared within the void volumes. Of the 1.45 × 10^6 and 2.31 × 10^6 cpm applied to the columns in Figures 6A and B, respectively, 1.02 × 10^6 cpm (70%) and 1.78 × 10^6 cpm (77%) were recovered in acid-insoluble form, indicating that the hydrolysis of the aminoacyl residues from tRNA was not severe in spite of the slightly alkaline pH of buffer C; it should, however, be realized that the immediate addition of sodium acetate buffer (pH 4.5) to the collected fractions kept exposure to pH 8.5 to a minimum of 1.5 hr or less, in the cold. Hydrolysis of the aminoacyl residues at the alkaline pH necessary for the formation of the borate complex with uncharged tRNA was originally envisaged as one major problem of the separation technique described here. This problem may still be serious if much larger quantities of aminoacylated tRNA than those described here are to be separated. In this case, the time necessary for filtration on larger gel beds will be longer and exposure to the pH 8.5 buffer might then be excessive; however, use of the more stable and easily accessible N-acetyl-aminoacyl-tRNAs (Haenni and Chapeville, 1966; Lapidot *et al.*, 1967) might circumvent this problem; subsequent removal of the N-acetylaminoacyl residues should be possible under the standard chemical stripping conditions or enzymatically with peptidyl-tRNA hydrolase (Kössel, 1970).

The radioactivities per A_{260} unit before application to the column were estimated to be 55,100 cpm per A_{260} unit for [³H]glutamyl-tRNA and 82,700 cpm per A_{260} unit for [³H]lysyl-tRNA. Monitoring of the peaks I from Figures 6A and 6B, respectively, indicated 340.000 cpm per A_{260} unit for purified [³H]glutamyl-tRNA and 726.000 cpm per A_{260} unit for purified [³H]lysyl-tRNA, from which purification factors of 6.2 and 8.8, respectively, can be deduced. (Due to spontaneous hydrolysis of the aminoacyl residues, purification factors tend to decrease remarkably during further work-up if the ratios of radioactivity to A_{260} units are used to express the purification factors.)
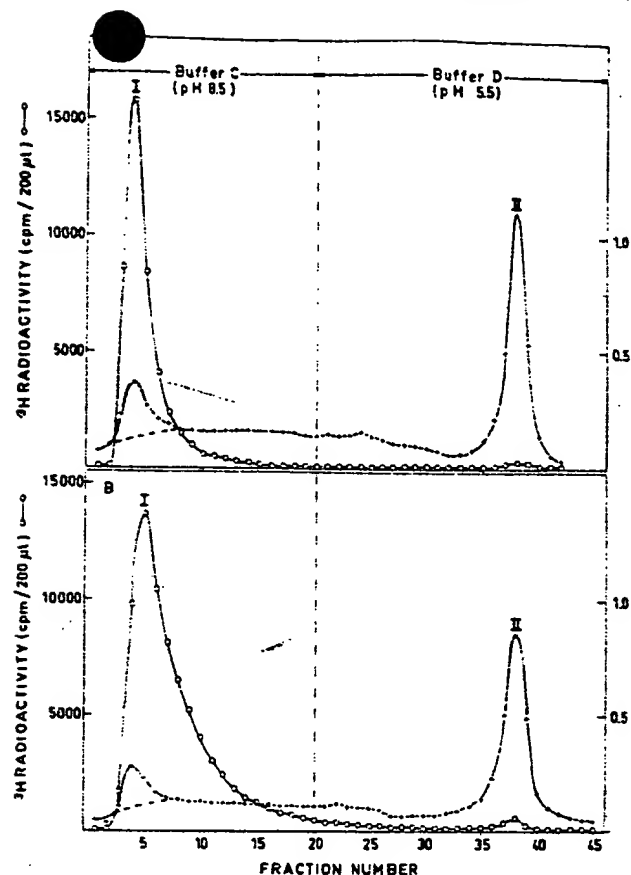

FIGURE 6: Purification of *E. coli* aminoacyl-tRNA by column chromatography on borate gels. (A) Separation of a tRNA mixture containing 674 pmol of [³H]glutamyl-tRNA (1.6%) in a total of 26.3 A_{260} units of tRNA; (B) separation of a tRNA mixture containing 1030 pmol of [³H]lysyl-tRNA (2.4%) in a total of 27.9 A_{260} units of tRNA. Elution buffers were applied as indicated by the arrows. Fractions of 4.6-ml volume were collected every 5 min in tubes containing 200 μl of 1 M sodium acetate, pH 4.5, in order to neutralize the eluent immediately. Aliquots of 200 μl were assayed for acid-insoluble radioactivity (O). Absorbancy of the individual fractions was followed at 260 nm (+); the absorbancy background of the elution buffer alone (—) is not subtracted in the figures.

Aminoacylation of the purified glutamyl-tRNA species obtained from Figure 6A as compared to the starting material and with the material eluted with buffer D was tested (Figure 7). When [¹⁴C]glutamic acid was used for the aminoacylation reaction, a large increase of incorporation was observed with material from peak I of Figure 6A compared to the starting material and to material from peak II of Figure 6A. The residual glutamic acid acceptor activity observed in material from peak II (Figure 7A, lower curve) probably arises from uncharged tRNA^{Glu}, present during the column run due to incomplete aminoacylation and/or to spontaneous hydrolysis of the glutamyl residues from tRNA. As a control, aminoacylation with [¹⁴C]lysine was tested with the same fraction (Figure 7B). As expected, the relative acceptor activity in this case increased slightly when material from peak II of Figure 6A was compared to the starting material, and reduced acceptor activity was observed with material from peak I of the same figure (see lower curve of Figure 7B). Though the relative acceptor activities of Figure 7B fit the expectations grossly, the residual lysine acceptor activity of material from peak I Figure 6A is difficult to explain; possible explanations are (the presence of nonradioactive lysyl-tRNA in peak I Figure 6A, due to the presence of nonradioactive lysine durin

he original aminoacylation reaction and/or (b) the presence of RNA in the aminoacyl-tRNA synthetase mixtures. Further investigation in order to clarify this problem seems necessary, especially as complete purification of the aminoacylated species should theoretically be possible by this method. It should be pointed out that the separation technique for tRNA described here in principle should also work with the borate-substituted cellulose described by Gilham and coworkers (Weith *et al.*, 1970; Rosenberg and Gilham, 1971).

Conclusion

The capacities of the borate gels seem to depend on the nature, particularly on the chain length, of the material to be separated. Thus, 680 A_{260} units of the mononucleotide 5'-riboadenylic acid, in contrast to only 80 A_{260} units (or less) of tRNA could be absorbed on a 25-ml column (0.5% boron content). Figure 3 indicates that the capacity for oligonucleotides ranges between the limits observed for mononucleotides and tRNA, although an exact estimation was not carried out for oligonucleotides. The differences in capacity probably arise from the reduced accessibility of those borate groups in interior parts of the gel matrix with increasing molecular weight or molecular diameter of the compounds to be absorbed. Further evidence for nonaccessible borate groups of the matrix is obtained from elementary analysis; corresponding to the average of 0.5% boron observed, roughly 5×10^{-4} equiv of cis diol-containing compounds should be absorbed per gram of dry matrix, corresponding to about 6 ml of gel. The capacity observed for mononucleotides indicates that about 1×10^{-5} equiv per gram of dry matrix can be absorbed, which corresponds to 2% of the theoretical value. Estimating 80 A_{260} units of tRNA absorbed per 25 ml of borate gel as roughly 2×10^{-7} equiv of 3'-terminal cis diol groups, it is evident that the effective capacity of the borate gel matrix is four orders of magnitudes below the theoretical value for tRNA (4×10^{-6} equiv/g of dry matrix), indicating that the macromolecule probably does not penetrate the interior regions of the matrix and/or that only the borate groups at the surface are efficient in the absorption process. In this connection it is interesting to note that the pore size of the borate gel probably can be varied simply by variation of the concentration of the cross-linking reagent during polymerization of the matrix. This possibility would allow construction of borate gels in which molecular sieve effects are superimposed on the borate–diol interaction. Thus, in contrast to the borate-containing cellulose, the exclusion limits of the borate gel described here should be adjustable to the separation problems to be solved. Further studies, however, will be necessary to verify this possibility.

Additional reasons for the reduced capacities for tRNA (and perhaps oligonucleotides) may be sought in the kinetics for the absorption process: it seems not unlikely that the cis diol group of a more complex molecule as tRNA is more or less hidden in a groove of the macromolecule, into which only the aminoacyl residues and part of the aminoacyl-tRNA synthetases fit in a highly specific way, whereas borate groups of a gel matrix reach the cis diol groups much less frequently. The observation that tRNA is only absorbed in the presence of 0.1 M magnesium suggests that a certain tertiary structure, probably one in which the cis diol group is more exposed, is responsible for the interaction with the borate groups of the gel.

It should finally be pointed out that from its chemical constitution the dihydroxyboryl-substituted methacrylic
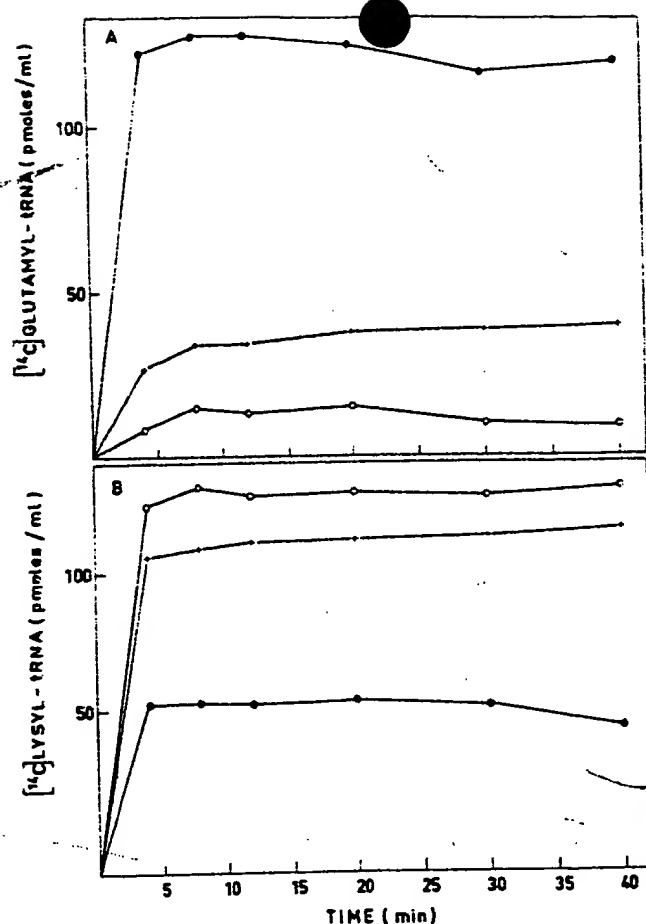

FIGURE 7: Aminoacylation of tRNA from peak 1 of Figure 6A with [^{14}C]glutamic acid (A) and [^{14}C]lysine (B). After stripping in 1 M Tris·HCl buffer (pH 8.8) for 30 min and concentration as described under Materials and Methods, aminoacylation was carried out according to Muench and Berg (1966) in total volumes of 200 μl containing 0.2 A_{260} unit of the individual tRNA fractions and 0.7 nmol of [^{14}C]amino acid in each tube. Aliquots of 30 μl were withdrawn at the times indicated and counted for acid-insoluble radioactivity. The individual curves represent experiments with material from peak I of Figure 6A (●) and as controls from peak II of Figure 5 (crude tRNA mixture) (+), and from peak II of Figure 6A (O). Control experiments without RNA (not shown) yielded 0.3 pmol (A) and 0.02 pmol (B), which were subtracted from the data shown.

acid polymer described in this article appears to be more resistant to degradation by heat or by microorganisms as compared to borate-substituted celluloses. The latter aspect seems of special importance for the practical usefulness of the gel.

References

Haenni, A. L., and Chapeville, F. (1966), *Biochim. Biophys. Acta 114*, 135.

Hurwitz, J., and Novogrodsky, A. (1968), *Methods Enzymol. 12B*, 207.

Khym, J. X. (1967), *Methods Enzymol. 12A*, 93.

Kössel, H. (1970), *Biochim. Biophys. Acta 204*, 191.

Kössel, H., and Roychoudhury, R. (1971), *Eur. J. Biochem. 22*, 271.

Kössel, H., Roychoudhury, R., Fischer, D., and Otto, A. (1973), *Methods Enzymol.* (in press).

Lapidot, Y., De Groot, N., and Fry-Shafrir, I. (1967), *Bio-*

chim. Biophys. Acta 14●●2.

Mans, R. J., and Novelli, G. D. (1961), Arch. Biochem. Biophys. 94, 48.

Muench, K. H., and Berg, P. (1966), in Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper and Row, p 375.

Ralph, R. K., and Khorana, H. G. (1961), J. Amer. Chem. Soc. 83, 2926.

Rosenberg, M., and Gilham, P. T. (1971), Biochim. Biophys. Acta 246, 337.

Roychou●●y, R. (1972), J. Biol. Chem. 247, 3910.

Schott, H. (1972a), Angew. Chem. 84, 819.

Schott, H. (1972b), Angew. Chem., Int. Ed. Engl. 11, 824.

Waters, L. C., and Novelli, G. D. (1968), Biochem. Biophys Res. Commun. 32, 971.

Weith, H. L., Wiebers, J. L., and Gilham, P. T. (1970) Biochemistry 9, 4396.

Yang, W.-K., and Novelli, G. D. (1968), Proc. Nat. Acad Sci. U. S. 59, 208.

Zubay, G. (1962), J. Mol. Biol. 4, 347.

Periodate Oxidation and Amine-Catalyzed Elimination of the Terminal Nucleoside from Adenylate or Ribonucleic Acid. Products of Overoxidation[†]

Mayo Uziel

ABSTRACT: The kinetic constants of the periodate oxidation of compounds derived from the amine-catalyzed elimination of phosphoric esters differ for mononucleotide and RNA substrates. Both substrates show kinetic curves similar to that for glycol oxidation. The reaction is first order in periodate, and 2 mol of formic acid is produced at the same rate as the free base. CO_2 is formed from overoxidation of formate or the three-carbon fragment from the elimination step. Our data do not support Rammler's hypothesized mechanism for elimination and overoxidation. The evidence suggests that ether cleavage (C-4'-O-C-1') occurs before overoxidation.

Ribonucleic acid can be sequentially degraded by the combined action of periodate and primary amines on terminal nucleotides, which leads to cleavage of the phosphoric ester linkage (Khym and Cohn, 1961; Whitfield and Markham, 1953; Uziel and Khym, 1969). In the presence of excess periodate, the nucleoside fragment is further degraded to free base (Neu and Heppel, 1964). The compound formed immediately upon elimination of the phosphoryl ester has not been identified, although there has been speculation on its structure (Rammler, 1971). As this product is also the substrate for the overoxidation,[1] discussions of elimination and overoxidation are not always mutually exclusive. For example, Khym (1963) has isolated an amine adduct (18, Figure 2) in the elimination reaction, and Rammler (1971) has used this observation to support his mechanism for elimination and overoxidation.

The overoxidation process is defined as the requirement for a large excess of periodate to cleave the remaining carbohydrate material to release the base (Neu and Heppel, 1964; Tankó et al., 1967). The substrate for this reaction is the amine-catalyzed elimination product. The products of the overoxidation include formate and CO_2, an observation which has provided the basis for the description by Rammler (1971) of a novel mechanism for the elimination and overoxidation. The author has examined the chemistry of these reactions with a view toward optimizing the overoxidation and elimination conditions for sequential degradation of RNA. This paper concerns the overoxidation step; the detailed kinetics and mechanism of elimination will be discussed in a separate publication.

Materials and Methods

All reagents were of the highest purity available. $NaIO_4$ was purchased from Matheson Coleman Bell Co., ornithine was obtained from Calbiochem, and the RNA was prepared by phenol extraction from Escherichia coli B. Purification of the tRNA fraction was by DEAE chromatography (Nishimura et al., 1967). Carbon dioxide formation was measured by a nitrogen gas transfer of the released gas to a standardized solution of barium hydroxide, followed by titration of the excess alkali with standard HCl. Bases were measured spectrophotometrically after cation-exchange chromatography at pH 5.2 (Uziel et al., 1968; Uziel et al., 1971). Cytidine and other nucleoside derivatives were measured on a 15-cm column of cation-exchange resin according to Table V in Uziel et al. (1968). Optical rotations were measured in 2-dm cells in a Rudolph polarimeter at room temperature (23°). Phosphate was measured by the technique of Hurst and Becking (1963).

[1] Overoxidation is defined as a requirement for excess periodate at elevated temperatures to release the purine or pyrimidine attached to the elimination product. Since we have been unable to isolate an intermediate step in the conversion of the elimination product to the overoxidation substrate, both these terms are used to refer to the compounds present in solution after elimination, when no excess periodate is present. They are used individually to emphasize the properties of the elimination reaction and the overoxidation reaction, respectively.